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## TURFGRASS SCIENCE

### Cytokinin Effects on Creeping Bentgrass Responses to Heat Stress: I. Shoot and Root Growth

Xiaozhong Liu, Bingru Huang\*, and Gary Banowetz

#### ABSTRACT

Heat stress injury may involve inhibition of cytokinin biosynthesis in roots. The objective of this study was to examine whether application of a synthetic cytokinin, zeatin riboside (ZR), to the root zone would enhance tolerance of creeping bentgrass (*Agrostis palustris* L.) to high soil temperature or in combination with high air temperature. Grasses were exposed to three air and soil temperature regimes for 56 d in growth chambers: (i) optimum air and soil temperature (control), 20/20°C; (ii) optimum air but high soil temperature (20/35°C); and (iii) high air and soil temperatures (35/35°C). Four concentrations (0.01, 0.1, 1, and 10  $\mu\text{mol}$ ) of ZR or water (control) were injected into the 0–5 cm root zone on the day before heat stress (0 d) and 14 d after. Turf visual quality, canopy net photosynthetic rate ( $P_n$ ), leaf photochemical efficiency (Fv/Fm), and vertical shoot extension rate decreased, whereas root mortality and root electrolyte leakage increased at 20/35 and 35/35°C, and to a greater extent at 35/35°C. Applications of 1 and 10  $\mu\text{mol}$  ZR mitigated heat stress injury to shoots and roots during most of the experimental period, with 10  $\mu\text{mol}$  ZR being more effective when applied at either 0 or 14 d of heat stress. Application of 0.1  $\mu\text{mol}$  ZR was less effective than 1 and 10  $\mu\text{mol}$  ZR. Application of 0.01  $\mu\text{mol}$  ZR had no effects on shoot and root responses to high soil temperature alone or combined with high air temperature. Endogenous cytokinin content in both shoots and roots increased with the application of 1 and 10  $\mu\text{mol}$  ZR. These results demonstrated that applying ZR at 1 or 10  $\mu\text{mol}$  concentration to the root zone could alleviate heat stress injury of creeping bentgrass.

THE OPTIMUM TEMPERATURES for shoot and root growth of cool-season grasses are 10 to 24°C (Beard, 1973). However, air and soil temperatures during mid-summer are often supraoptimal. Previous studies suggested that high soil temperature is more detrimental than high air temperature, particularly to root growth (Beard and Daniel, 1966; Skene, 1975; Aldous and Kaufmann, 1979; Kuroyanagi and Paulsen, 1988; Xu and Huang, 2000a,b). In contrast, reducing soil temperature at high air temperature improves shoot and root growth. Although extensive research has been done on physiological responses of turfgrasses to heat stress (DiPaola and Beard, 1992), the physiological mechanisms underlying cool-season grass responses to soil temperatures are not well understood. Cytokinins, hormones produced mainly in roots, may regulate plant responses to high soil temperature.

Cytokinin metabolism of roots is sensitive to heat stress. Two minutes of heat shock to the roots of *Nicotiana rustica* L. and *Phaseolus vulgaris* L. reduced cytokinin levels in both shoots and roots (Itai et al., 1973). Treatment with high air and soil temperatures (45/45°C) for 5 h reduced the levels of zeatin riboside (ZR) and isopentenyl adenosine in roots of both *Phaseolus acutifolius* A. Gray and *P. vulgaris* (Udomprasert et al., 1995). Heat stress also reduced ZR content in winter rape (*Brassica napus* L.) (Zhou and Leul, 1999).

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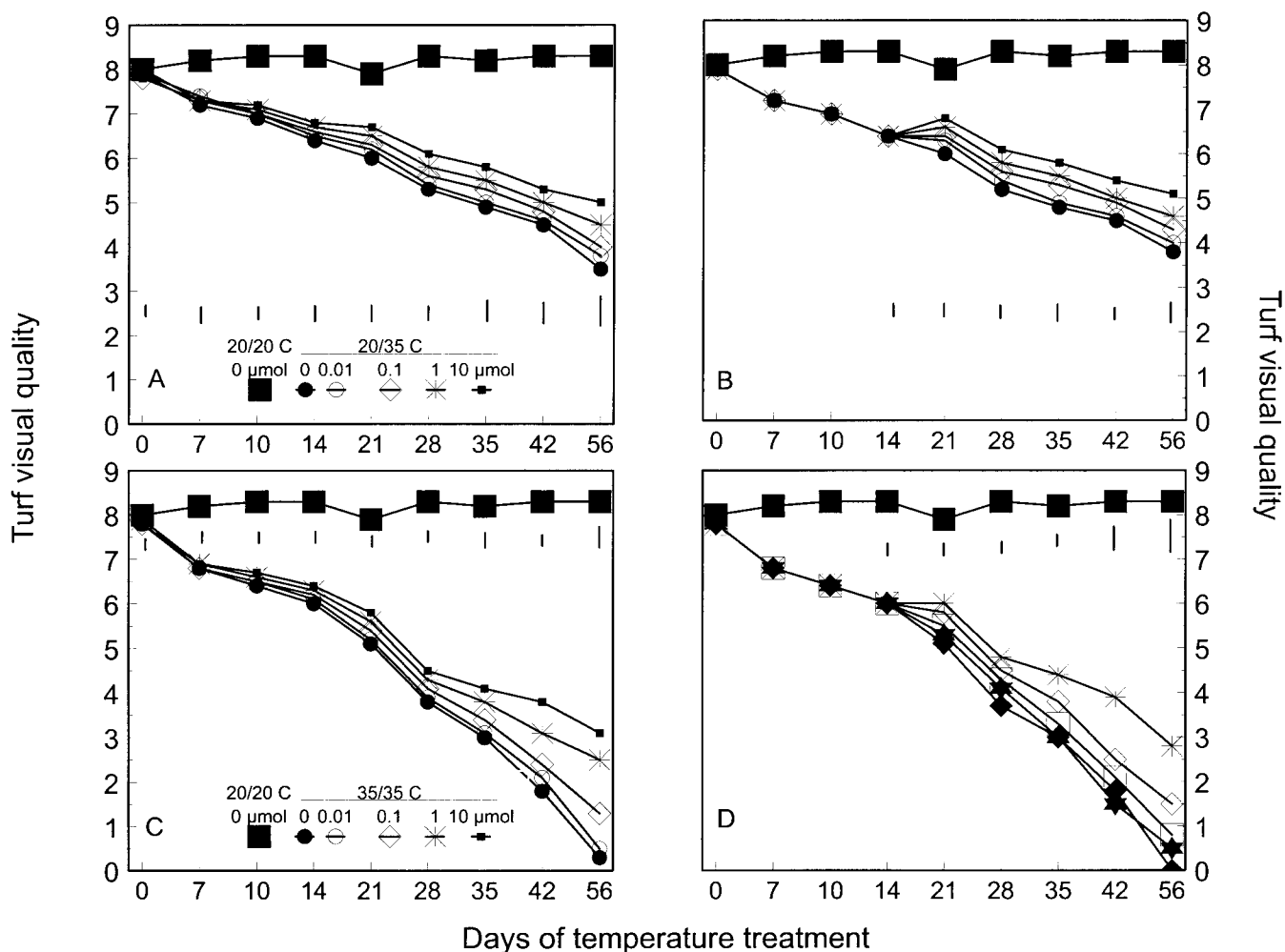


Fig. 1. Visual turf quality of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

Exogenous cytokinins have been used to alleviate heat stress injury. Applying kinetin reversed heat shock injury in wheat (*Triticum aestivum* L.) root tips (Skogqvist and Fries, 1970; Skogqvist, 1974). Applying benzyladenine (BA) to *P. vulgaris* leaves helped maintain high chlorophyll content, delaying leaf senescence under normal environmental conditions (Adedipe et al., 1971). Applying the carbamide cytokinin 4PU30 promoted the recovery of *P. vulgaris* from water deficit and high temperature stress (Yordanov et al., 1997).

Little information is available on the potential effects of exogenous cytokinins in alleviating of heat stress injury in cool-season grasses, including creeping bentgrass. This study was designed to investigate whether application of the cytokinin ZR to the root zone could alleviate heat injury in shoots and roots of creeping bentgrass induced by high soil temperature or in combination with high air temperature.

## MATERIALS AND METHODS

Sod pieces of 3-year-old 'Penncross' creeping bentgrass were collected from field plots and transplanted into clear bottom-sealed polyethylene tubes (7.6 cm in diameter and 60

cm in length, with holes pierced at the bottom for drainage) filled with a mixture of 10% fritted clay (Profile, AIMCOR, Deerfield, IL) and 90% sand (80% of medium to coarse sand  $-0.25$  to  $1.0$  mm and 20% fine sand  $-0.15$  to  $0.25$  mm). The clear polyethylene tubes were placed in opaque polyvinyl chloride (PVC) tubes of the same diameter and length, which were installed vertically in water baths with the lower open end exposed from the bottom of the water bath for drainage (Xu and Huang, 2001). These were designed in a way that enabled plant growth in well-drained soil in polyethylene bags, while soil temperature was controlled at a constant, predetermined level.

Grasses were maintained in growth chambers for about 2 months with a temperature regime of 20/20°C (day/night),  $600 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation at the canopy level, 60% relative humidity, 385 ppm  $\text{CO}_2$ , and 14 h of photoperiod. Turf was mowed daily at 4 mm height with an electric clipper and irrigated daily until water drained freely from the bottom of tubes. Each container received a weekly application of 20 mL full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) to maintain adequate nutrient level.

## Treatments and Experimental Design

Grasses were exposed to three constant day/night temperature regimes for 56 d: (i) control: low air and soil temperatures

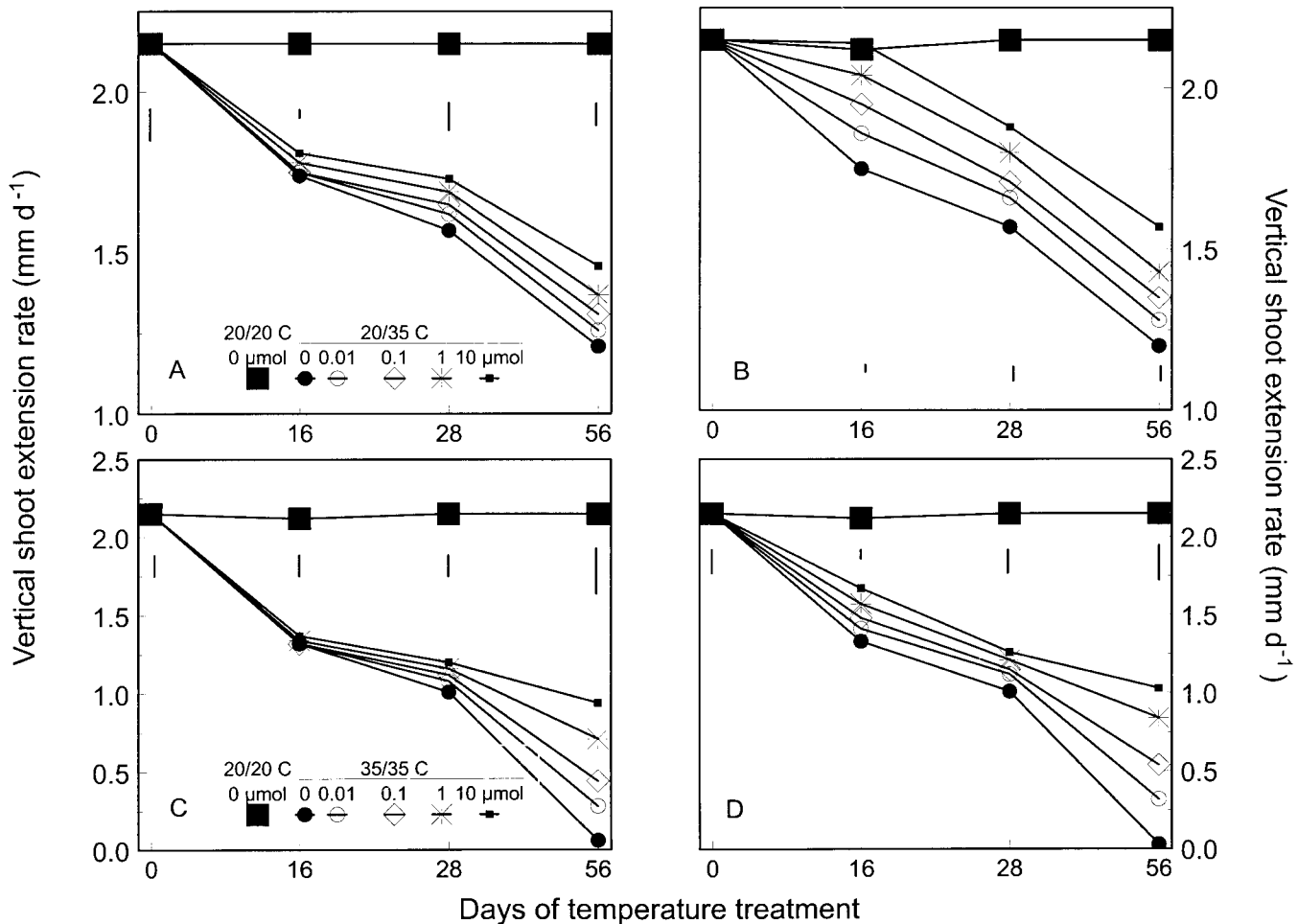


Fig. 2. Vertical shoot extension rate (VSER) of creeping bentgrass as affected by exogenous zeatin riboside (ZR) applications at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

(20/20°C); (ii) high soil temperature with optimum air temperature (20/35°C); and (iii) high air and soil temperatures (35/35°C). For the 20/35°C regime, air temperature was controlled at 20°C by the temperature controller in the growth chamber, and soil temperature was controlled at 35°C by circulating warm water around PVC tubes placed vertically in water baths using an immersion circulating heater (Fisher Scientific Inc., Pittsburgh, PA). In the 20/20°C and 35/35°C regimes, both air and soil temperatures were controlled by the temperature controller in the growth chambers.

On the day before high temperature treatments were imposed (0 d) and 14 d of treatments, 50 ml of water or 0.01, 0.1, 1.0, and 10 μmol ZR were injected into the 0–5 cm root zone at 1, 3, and 5 cm from the soil surface in each tube with hypodermic syringes. This range of ZR concentration has been found to be effective to alleviate heat injury in other species (Skogqvist and Fries, 1970; Skogqvist, 1974; Yordanov et al., 1997).

The experimental design was a completely randomized split-plot design with temperature as the main plot and ZR concentration as the subplot in four replicates for each measurement. Each temperature treatment was replicated four times in four different growth chambers and water baths. The ZR treatments were arranged randomly in each temperature treatment. Effects of temperature, ZR concentration, and their interaction were determined by analysis of variance according to the general linear model procedure of Statistical Analysis System (SAS Inc., Cary, NC). Differences between

treatment means were separated by the least significance difference (LSD) test at the 0.05 level.

### Measurements

Turf visual quality, leaf photochemical efficiency ( $F_v/F_m$ ) and canopy net photosynthetic rate ( $P_n$ ) were measured non-destructively at various times during the experiment. Turf visual quality was evaluated as the integration of shoot density, uniformity, and color and ranked from 0 to 9, where 0 is the worst and 9 is the best. Leaf photochemical efficiency was estimated by measuring leaf chlorophyll fluorescence ( $F_v/F_m$ ) with a plant efficiency analyser (Hansatech Instrument LTD, Kings Lynn, England). Canopy net photosynthetic rate ( $P_n$ ) was measured with the Li-COR 6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE). Vertical shoot extension rate was estimated by measuring the difference in mean canopy shoot height before and after cutting at 2-day intervals.

Roots were sampled biweekly for measurements of mortality and electrolyte leakage, an indicator of cell membrane integrity. They were washed free of sand and rinsed in deionized water. Root mortality was measured as dehydrogenase activity by the triphenyltetrazolium chloride (TTC) reduction method (Knievel, 1973). A 1 to 3 g sample of fresh roots was immersed in 20 ml of 0.6% TTC (dissolved in 50 mM phosphate buffer, pH 7.4) for 24 h at 30°C. Roots then were rinsed in distilled water and extracted twice in 95% ethanol. The

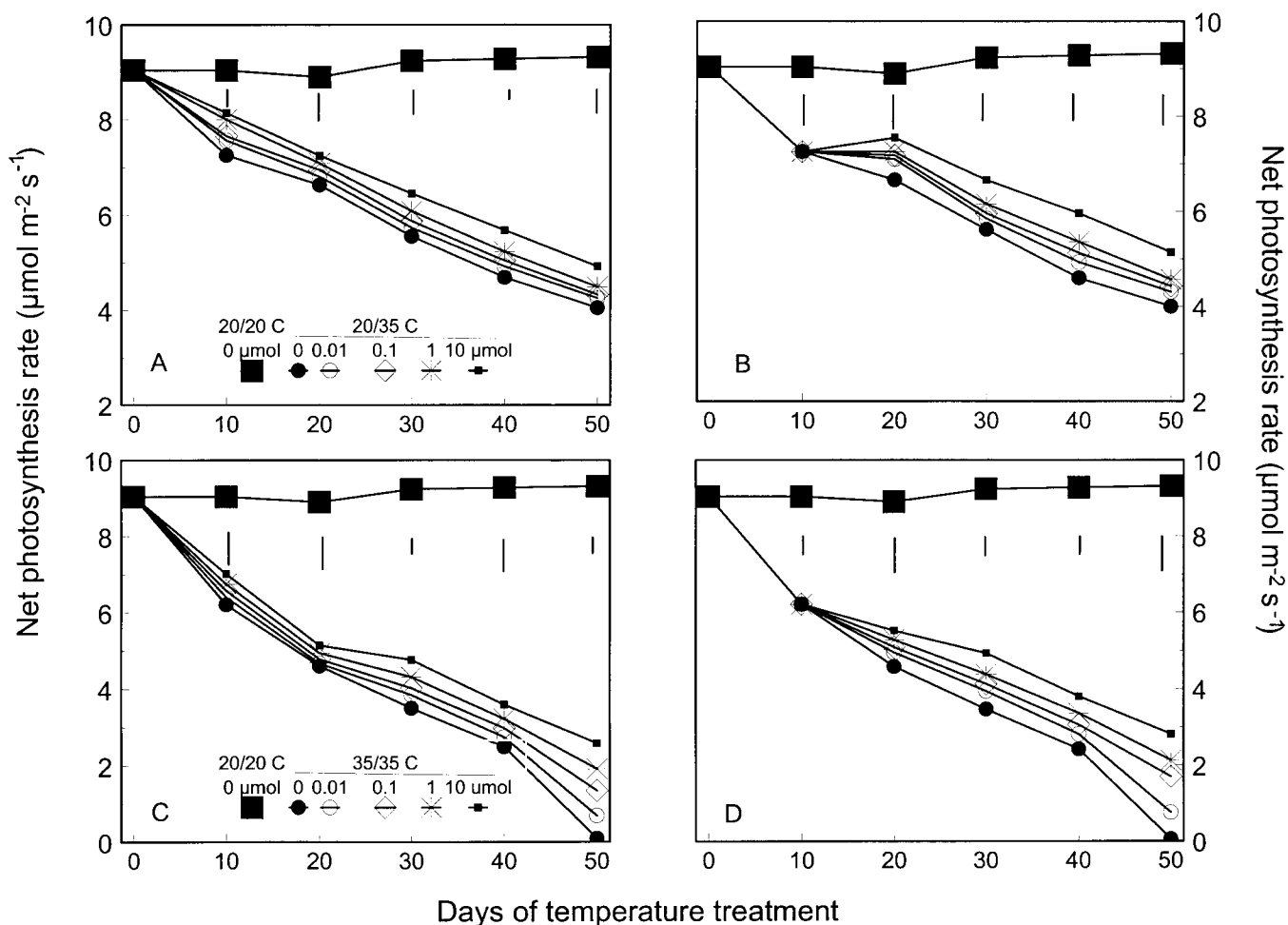


Fig. 3. Canopy net photosynthetic rate ( $P_n$ ) of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

absorbance of extractants in roots was measured at 490 nm with a spectrophotometer (Spectronic Instruments, Rochester, NY). For electrolyte leakage, a 0.1 g sample of roots was immersed in 20 ml deionized water and placed under vacuum for 15 min. Samples in solution were shaken on a shaker for 24 h. The conductivity of the solution was measured with a YSI Model 32 Conductivity Meter (Yellow Spring, OH). Then the tissues were killed by autoclaving at 140°C for 20 min. The conductivity of the dead tissues was measured. Relative electrolyte leakage was calculated as the percentage of conductivity of live roots over that of dead roots.

At various days after treatment, leaves and roots were sampled and frozen in liquid nitrogen and stored in plastic vials at  $-20^\circ\text{C}$  until analysis. Extraction of cytokinin and analysis of cytokinin followed the methods described in Trione and Syayavedra-Soto (1988). Cytokinins were quantified with monoclonal antibodies tzR3 (Trione et al., 1985) and iPA3 (Trione et al., 1987) in a fluorescence ELISA. These antibodies permitted the quantification of zeatin, dihydrozeatin, and isopentenyl adenine free bases, ribosides, and 9-glucosides.

## RESULTS

### Visual Turf Quality

Turf quality of plants with or without ZR treatment decreased significantly below the control level (20/

20°C), beginning at 7 d under high soil temperature (20/35°C) (Fig. 1A, B) and high air/soil temperatures (35/35°C) (Fig. 1C, D). At the end of the experimental period (56 d), turf quality had decreased to 3 to 5 at 20/35°C and 0 to 3 at 35/35°C, respectively, depending on ZR concentrations.

The reduction in turf quality for plants untreated with ZR was 49% at 35/35°C and 32% at 20/35°C averaged over the experimental period. The decrease in turf quality for ZR-treated plants was 43% at 35/35°C and 27% at 20/35°C when ZR was applied at 0 or 14 d of heat stress. Grasses treated with 10  $\mu\text{mol}$  ZR had the highest turf quality, followed by those treated with 1  $\mu\text{mol}$  ZR, after 21 d of both temperature treatments. Applying 0.1  $\mu\text{mol}$  ZR did not have significant effects on turf quality at 20/35°C but significantly increased turf quality after 42 d at 35/35°C. Applying 0.01  $\mu\text{mol}$  ZR had no effects on turf quality at either 20/35°C or 35/35°C.

### Vertical Shoot Extension Rate (VSER)

Vertical shoot extension rate of plants without ZR treatment decreased significantly below the control (20/20°C) level, beginning at 16 d of 20/35°C (Fig. 2A, B) and 35/35°C (Fig. 2C, D). The VSER increased at 28

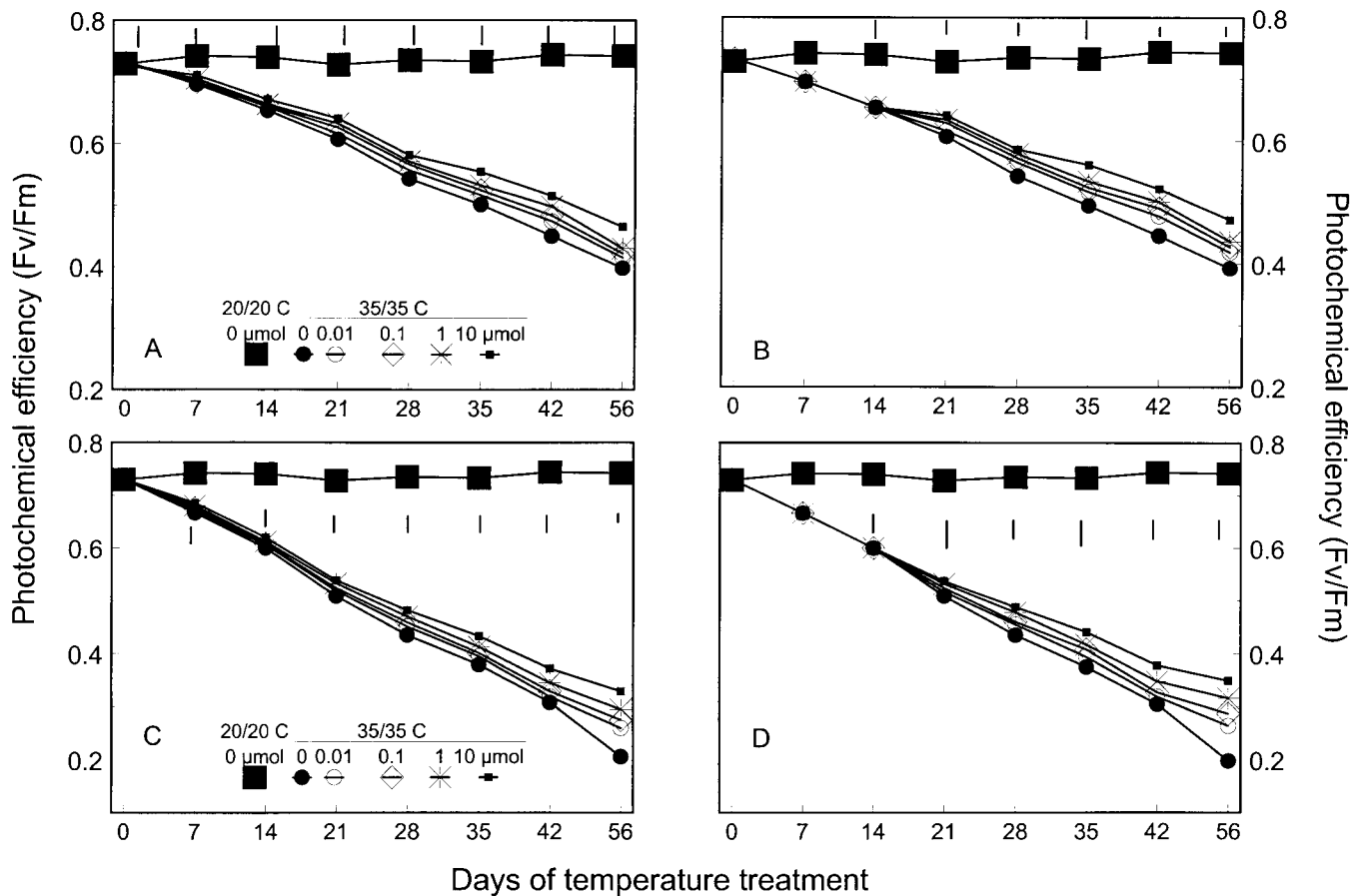


Fig. 4. Leaf photochemical efficiency (Fv/Fm) of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

and 56 d of heat stress with application of 10, 1, and 0.1  $\mu\text{mol}$  ZR at 0 or 14 d of 20/35°C and 35/35°C. Grasses treated with 10, 1, and 0.1  $\mu\text{mol}$  ZR at 0 d of heat stress had significantly higher VSER than untreated plants after 16, 16, and 28 d of 20/35°C (Fig. 2A), respectively, and after 28, 28, and 56 d of 35/35°C (Fig. 2C), respectively. The 0.01  $\mu\text{mol}$  ZR applied at 0 d did not significantly affect VSER during most of stress periods under either 20/35°C or 35/35°C. The VSER increased by 15% when ZR was applied at 14 d (Fig. 2B, D) and 7% when ZR was applied at 0 d (Fig. 2A, C) at 20/35°C and 35/35°C, averaged over the different ZR concentrations. Application of ZR at all concentrations at 14 d were effective in increasing VSER under both high temperature regimes, except at 28 d at 35/35°C where 0.01 and 0.1  $\mu\text{mol}$  ZR had no effect.

#### Net Photosynthetic Rate

Net photosynthetic rate ( $P_n$ ) at both 20/35°C (Fig. 3A, B) and 35/35°C (Fig. 3C, D) decreased significantly below the control level (20/20°C), beginning at 10 d of the experimental period, regardless of ZR treatment. At the end of the experiment,  $P_n$  value of untreated plants ranged from 45 to 55% lower than the control at 20/35°C and from 80 to 100% lower at 35/35°C, depending on ZR application rates.

The  $P_n$  values of plants treated with 10, 1, and 0.1

$\mu\text{mol}$  ZR at 0 d of heat stress were greater than those of untreated plants, after 10, 10, and 30 d of 20/35°C, respectively, and after 10, 30, and 30 d of 35/35°C, respectively. Grasses treated with 10, 1, and 0.1  $\mu\text{mol}$  ZR at 14 d of 20/35°C had higher  $P_n$  than untreated plants after 20 d; with applications at 14 d of 35/35°C, increasing  $P_n$  was observed after 20 d with 10 and 1  $\mu\text{mol}$  ZR and after 30 d with 0.1  $\mu\text{mol}$ . The 0.01  $\mu\text{mol}$  ZR applied at 0 or 14 d had no significant effects on  $P_n$  at 20/35°C but significantly increased  $P_n$  at 50 d of 35/35°C.

#### Leaf Photochemical Efficiency (Fv/Fm)

Leaf photochemical efficiency (Fv/Fm) under both 20/35°C (Fig. 4A, B) and 35/35°C (Fig. 4C, D) significantly decreased to below the control level (20/20°C) beginning at 7 d, regardless of ZR treatment. Grasses treated with 10, 1, and 0.1  $\mu\text{mol}$  ZR at 0 d had significantly higher Fv/Fm than untreated plants, beginning at 21, 28, and 42 d, respectively, at both 20/35°C and 35/35°C. Applying 0.01  $\mu\text{mol}$  ZR at 0 d did not have significant effects on Fv/Fm at 20/35°C but significantly increased Fv/Fm at 56 d of 35/35°C. All concentrations of ZR applied at 14 d of heat stress were effective in enhancing Fv/Fm, starting at 28 d for 10 and 1  $\mu\text{mol}$ , 42 d for 0.1  $\mu\text{mol}$ , and 56 d for 0.01  $\mu\text{mol}$ , under both high temperature regimes.

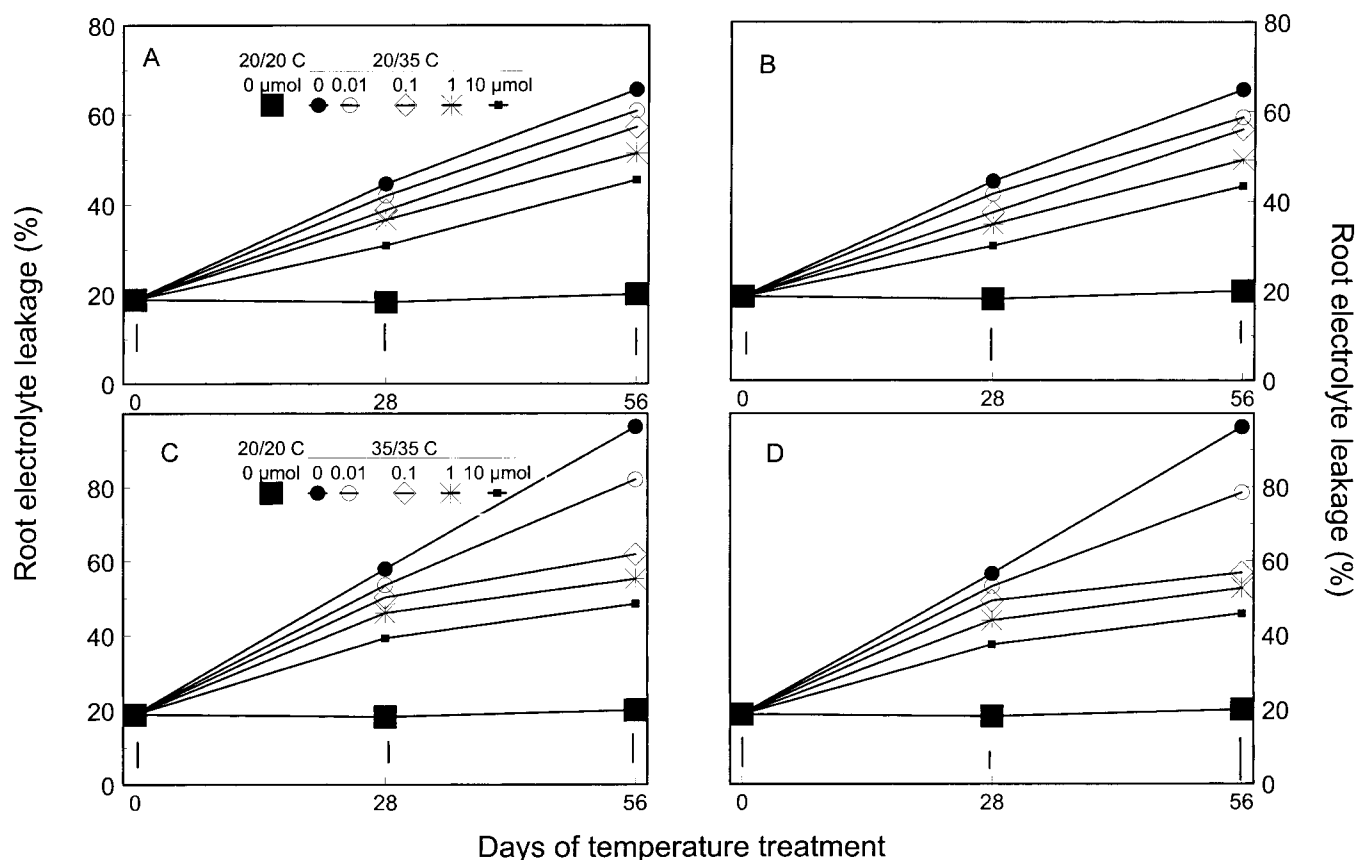


Fig. 5. Root electrolyte leakage (EL) of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

### Root Mortality and Electrolyte Leakage

Root mortality (Fig. 5) and electrolyte leakage (Fig. 6) of both ZR-treated and untreated plants increased above the control level at 28 and 56 d of 20/35°C (Fig. 5A, B; Fig. 6A, B) and 35/35°C (Fig. 5C, D; Fig. 6C, D).

Root mortality and EL were reduced by ZR application at all concentrations when applied at 0 or 14 d at 56 d of 20/35°C and 35/35°C; the reductions increased with ZR concentrations.

### Endogenous Cytokinin Contents

Endogenous cytokinin content, which was the sum of ZR and isopentenyl adenine free bases (iPA) content, in both shoots (Fig. 7) and roots (Fig. 8) of ZR-untreated plants were significantly lower at 20/35°C (Fig. 7A, B; Fig. 8A, B) and 35/35°C (Fig. 7C, D; Fig. 8C, D) than that at 20°C.

Cytokinin contents in both shoots and roots for plants treated with 10  $\mu\text{mol}$  ZR either at 0 or 14 d were significantly higher than those of untreated plants at 14, 28, and 56 d of 20/35°C and 35/35°C. Higher cytokinin contents were observed in shoots at 17, 28 and 56 d of 20/35°C and in roots at 28 and 56 d when 1  $\mu\text{mol}$  ZR was applied at 14 d. Shoot cytokinin content in plants at 17 d of 20/35°C treated with ZR at 14 d was even higher than those at control temperature untreated with ZR.

### DISCUSSION

Cytokinin level in both shoots and roots decreased under high soil or high air and soil temperatures, but increased when 10  $\mu\text{mol}$  ZR was applied to the root zone under either temperature regimes. Similar results have been reported by Udomprasert (1995) in other plant species. Applying ZR at the higher concentrations to the root zone alleviated the damage to both shoots and roots from prolonged exposure to high soil and/or air temperatures. These results suggested that the reduction in cytokinin content in roots could be involved in the damage to shoots and roots in creeping bentgrass from either high soil temperature alone or in combination with high air temperature. Roots are the primary sites of cytokinin synthesis and supply shoots with cytokinins (Henson and Wareing, 1976; Weiss and Vaadia, 1965; Forsyth and Van Staden, 1981) via translocation through the xylem (Carr and Burrows, 1966). Root die-back under high temperatures could result in limited cytokinin synthesis, which in turn, affect shoot growth and senescence (Adedipe et al., 1971).

The enhancement effects of ZR on vertical shoot extension rate at high temperatures increased with ZR concentrations, particularly when ZR was applied at 14 d of heat stress. Under either high soil or high air and soil temperature, 10  $\mu\text{mol}$  ZR treatment, the highest concentration tested in this experiment, was the most

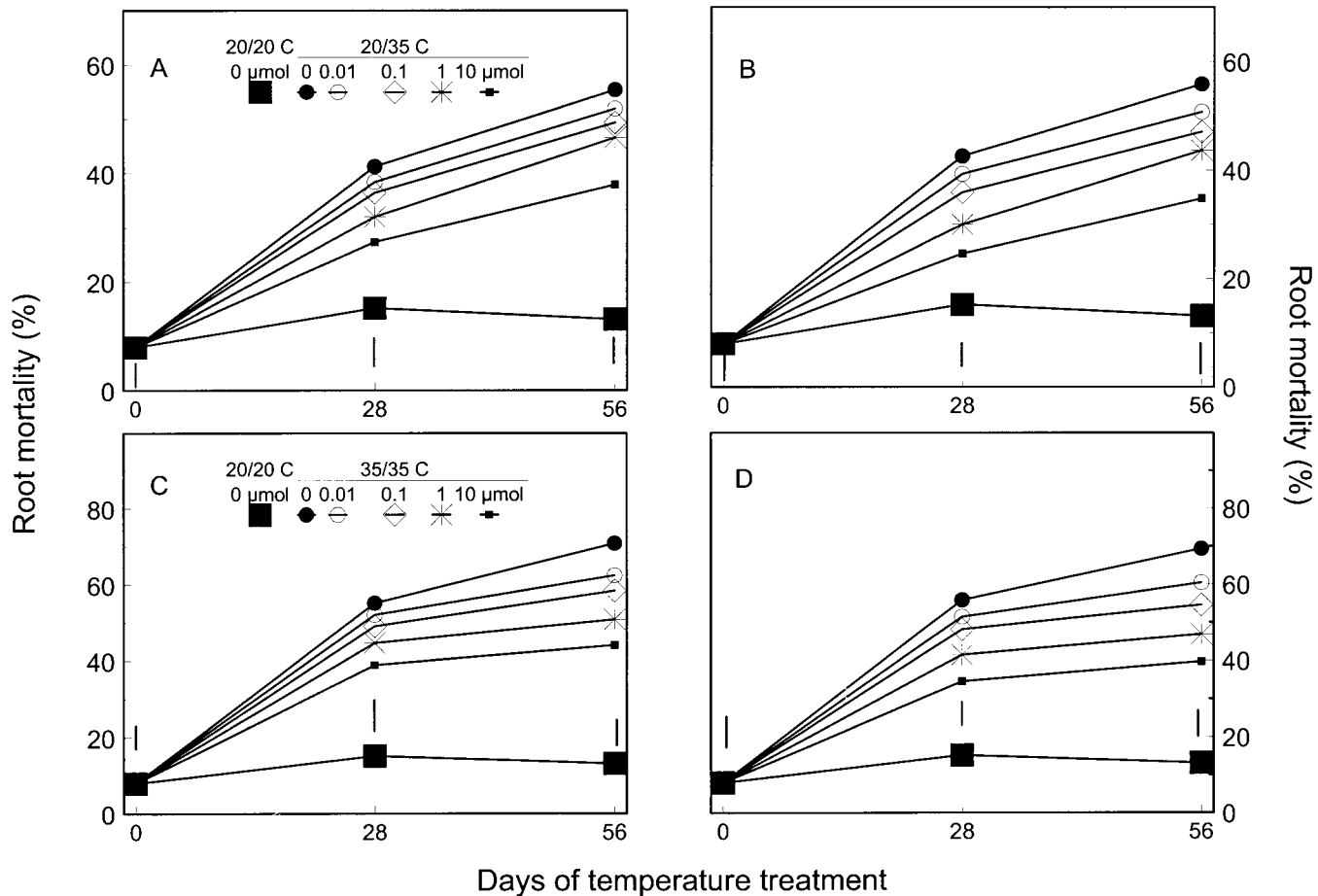


Fig. 6. Root mortality of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

effective in alleviating heat injury. Whether an even higher ZR concentration would be more effective is unknown and needs further investigation. However, high concentration of cytokinins can be inhibitory to shoot and root growth (Salisbury, 1985).

Higher concentrations of ZR (1 and 10 μmol) retarded the decline in photosynthetic rate for plants exposed to prolonged periods of high temperature stress, which could have been due to the effects of ZR in suppressing the decrease in photochemical efficiency and chlorophyll degradation (Liu and Huang, 2000). Photochemical efficiency and chlorophyll content are indicators of the integrity of the photosynthetic apparatus (Schreiber and Bilger, 1987; Swamy and Suguna, 1992). Our results suggested that ZR could alleviate the inhibitory effects of heat on photosynthesis by protecting the photosynthetic apparatus. Adedipe et al. (1971) reported that BA, another cytokinin, can help maintain high photosynthetic activity by retarding leaf senescence. However, applying 1 μmol ZR failed to increase photosynthetic rate in *P. acutifolius* and *P. vulgaris* (Udomprasert et al., 1995), and BA treatment also failed to increase photosynthesis in *P. vulgaris* (Carmi and Koller, 1978). Therefore, the effects of cytokinins on photosynthesis seem to be species and concentration dependent.

Vertical shoot extension rate was more responsive to ZR treatment than visual turf quality and photosynthesis, as demonstrated by the more pronounced and rapid increases in VSER after plants were treated with 10 μmol ZR, compared with their respective controls. Heat-stressed plants treated with 10 μmol ZR had VSER similar to the control level at 16 d of high temperatures or only 2 d after ZR application. This phenomenon can be explained by the major physiological function of cytokinins, which is promoting cell division and elongation (Salisbury, 1985). These processes are among the most sensitive to high temperature (Paulsen, 1994). Compared with shoot responses, root mortality and EL were more affected by ZR treatment, as demonstrated by the greater differences between the treated and untreated plants for roots than for shoot growth. This indicated that roots were more sensitive to ZR than shoots. This could be because cytokinin application to the root zone stimulated cytokinin synthesis in roots. How external application of ZR influences cytokinin levels in roots of creeping bentgrass is under investigation.

In summary, our study demonstrated that application of cytokinin at 1 or 10 μmol improved visual turf quality and vertical shoot growth under either high soil temperature alone or in combination of high air temperature.

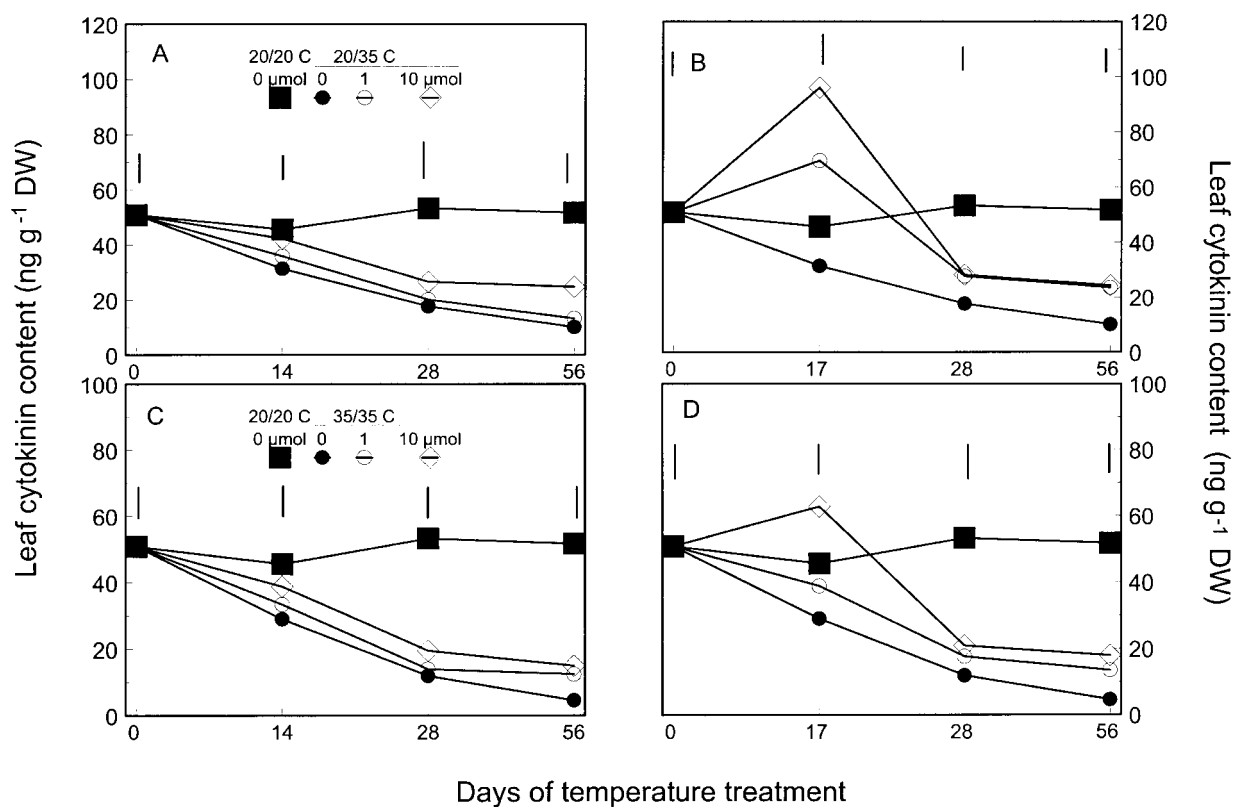


Fig. 7. Leaf cytokinin content of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

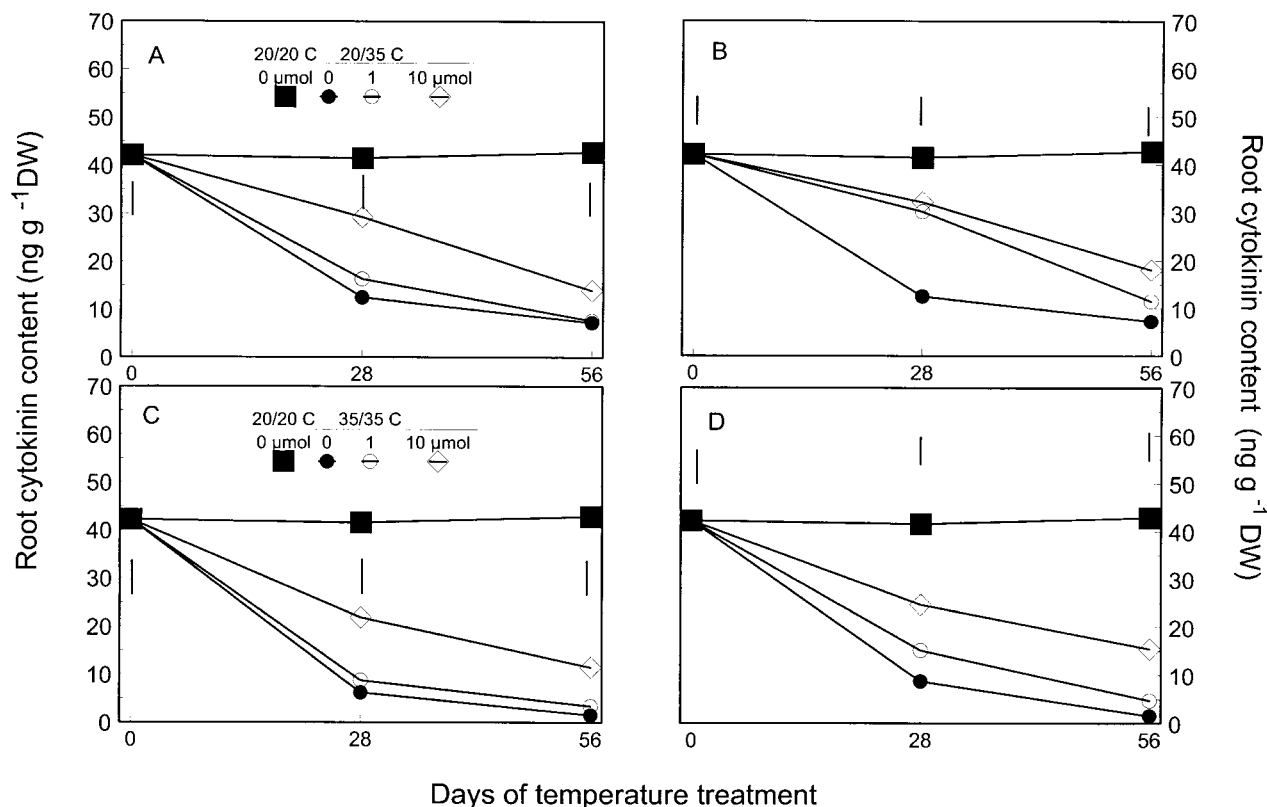


Fig. 8. Root cytokinin content of creeping bentgrass as affected by exogenous applications of zeatin riboside (ZR) at 0 d (A, C) and 14 d (B, D) of high soil temperature (20/35°C, A, B) and high air/soil temperatures (35/35°C, C, D). The symbols in B and D are the same as in A and C, respectively. Vertical bars indicate LSD values ( $P = 0.05$ ) for temperature and ZR concentration comparisons at a given day.

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